Use of dried blood spots for the determination of plasma concentrations of nevirapine and efavirenz

Wiete Kromdijk1*, Jan W. Mulder2, Hilde Rosing1, Patrick M. Smit2, Jos H. Beijnen1,3 and Alwin D. R. Huitema1

1Department of Pharmacy & Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands; 2Department of Internal Medicine, Slotervaart Hospital, Amsterdam, The Netherlands; 3Faculty of Science, Department of Pharmaceutical Sciences, Division of Biomedical Analysis, Section of Drug Toxicology, Utrecht University, Utrecht, The Netherlands

*Corresponding author. Tel: +31-20-5124737; Fax: +31-20-5124753; E-mail: wietekromdijk@hotmail.com

Received 25 October 2011; returned 18 December 2011; revised 29 December 2011; accepted 10 January 2012

Objectives: Plasma concentrations are frequently used for therapeutic drug monitoring of antiretroviral drugs. Dried blood spot sampling offers a patient-friendly and easy alternative to plasma sampling. However, dried blood spot concentrations are not necessarily equal to plasma concentrations and therefore the objective of this work was to establish the relationship between nevirapine and efavirenz dried blood spot and plasma concentrations to facilitate clinical implementation of dried blood spot sampling.

Methods: Paired dried blood spot and plasma samples were obtained from 40 HIV-infected patients on nevirapine and 40 on efavirenz treatment. All samples were analysed using validated HPLC–tandem mass spectrometry methods for the two matrices. Theoretical plasma concentrations were calculated from dried blood spot concentrations using the formula [dried blood spot concentration/(1 – haematocrit)] × fraction bound to plasma proteins = plasma concentration. Linear regression and Bland–Altman analysis were used to compare the two methods.

Results: Dried blood spot and plasma concentrations of nevirapine and efavirenz correlated well (r² = 0.867 and 0.972, respectively), although efavirenz dried blood spot concentrations were 39.8% (SD 7.1%) lower than plasma concentrations. Theoretical plasma concentrations (using patient-specific haematocrit) of nevirapine and efavirenz were similar to measured plasma concentrations, with a mean difference between the two methods of 0.29 mg/L (SD 1.35 mg/L) and 0.08 mg/L (SD 0.31 mg/L), respectively.

Conclusions: Dried blood spot concentrations of nevirapine and efavirenz were equal to plasma concentrations after correction for haematocrit and compound-specific plasma protein binding and can therefore be used in clinical practice.

Keywords: HIV, non-nucleoside reverse transcriptase inhibitors, therapeutic drug monitoring

Introduction

Approximately 33.3 million people are currently living with HIV.1 Since the introduction of combined antiretroviral treatment (cART) the morbidity and mortality of HIV-infected individuals has decreased dramatically. Guidelines from the European AIDS Clinical Society and the Department of Health and Human Services in the USA recommend HIV treatment with two nucleoside reverse transcriptase inhibitors combined with a protease inhibitor, a non-nucleoside reverse transcriptase inhibitor (NNRTI) or raltegravir.2,3 Unfortunately, the emergence of resistance and the occurrence of adverse events often limit the therapeutic success of antiretroviral drugs.

Plasma concentrations of the NNRTIs nevirapine and efavirenz are related to virological outcome and toxicity in HIV-infected patients.4,5 Therefore, therapeutic drug monitoring (TDM) of nevirapine and efavirenz is recommended in special populations (e.g. children and pregnant women) and for selected indications (such as certain drug–drug interactions) to minimize toxicity and maximize virological outcome.6 Currently, TDM is performed by measurement of plasma concentrations. However, venous sampling has several drawbacks, including the requirements for trained personnel and specialized equipment for sample processing. Additionally, measurement of trough levels is often advised for TDM, which is difficult to perform in an outpatient clinic with the current dosing regimens (once or twice daily) of antiretroviral agents.

Dried blood spots (DBS) offer a patient-friendly and easy alternative. DBS can be collected by patients with a simple finger prick, after which blood is collected on filter paper cards. This technique has previously been used for TDM of several other drugs.7 The cards can be stored at room temperature after air drying.
However, before the use of DBS can be implemented in clinical practice, a comparison between DBS and plasma concentrations is required since DBS concentrations are not necessarily equal to plasma concentrations. For instance, the fraction of analyte bound to plasma proteins (f_{pp}) and the haematocrit may influence DBS concentrations.\(^8,9\) Therefore, the objective of this work was to compare nevirapine and efavirenz concentrations between DBS and plasma using two previously validated HPLC–tandem mass spectrometry (MS/MS) methods developed for these matrices.\(^10,11\)

**Methods**

**Patients**

For this study, 40 HIV-infected patients on an efavirenz-based regimen and 40 HIV-infected patients on a nevirapine-based regimen were recruited from the outpatient clinic of the Slotervaart Hospital, Amsterdam, The Netherlands. The number of patients was based upon the guidelines for method comparison and bias estimation using patient samples from the CLSI.\(^12\) Besides the regimen, no further inclusion criteria were used. Patients were recruited between December 2010 and May 2011. Patient characteristics were obtained from patient files. Additionally, patients were asked for their timing of last efavirenz or nevirapine intake. This study was approved by the local ethics committee and informed consent was provided by each participant before blood sampling.

**Sampling**

DBS samples were obtained within 5 min after venepuncture, for the collection of EDTA whole blood, from each patient during regular visits to the outpatient clinic. Plasma samples were obtained from the EDTA whole blood samples after centrifugation and were stored at \(-20^\circ\)C until further analysis. After sterile cleaning of the skin, a lancet puncture was performed for DBS sampling. The first drop of blood was discarded and subsequent blood drops were collected on filter paper cards (Protein Saver 903 card, Whatman Nederland B.V., Den Bosch, The Netherlands). After overnight air drying, DBS samples were stored at room temperature in a foil bag with a desiccant package according to the manufacturer’s instructions pending further analysis.

**Bioanalysis**

Bioanalysis of nevirapine and efavirenz concentrations was performed using two previously validated HPLC–MS/MS assays for the determination of NNRTIs and protease inhibitors in plasma and DBS.\(^10,17\) In short, sample pretreatment of plasma consisted of protein precipitation with a mixture of methanol and acetonitrile (50:50, v/v) that contained internal standards. For DBS, the analytes were extracted from a 0.6 cm diameter punched-out disc, corresponding to \(\sim 15\) mL of whole blood, using a mixture of methanol, acetonitrile and 0.2 M zinc sulphate in water (1:1:2, v/v/v) with internal standards. Chromatographic separation was performed on a reversed-phase C18 column with a stepwise gradient using a mobile phase consisting of methanol with 10 mM ammonium acetate buffer pH 5 (35:65, v/v) and methanol. An API 3000 triple quadrupole mass spectrometer operating in the positive ionization mode was used for quantification of nevirapine and efavirenz (mass transitions of \(m/z\) 267/226 and 316/244, respectively). For nevirapine, most patients (\(n=28\)) were on the approved 600 mg once-daily dose regimen. For nevirapine, most patients (\(n=28\)) were on a once-daily 400 mg dose, 11 patients were on the approved 200 mg twice-daily dose and 1 patient was on a once-daily 500 mg dose. The most commonly used nucleoside analogue backbone was tenofovir/emtricitabine (\(n=58\)). Other nucleoside backbone were lamivudine/tenofovir (\(n=5\)), abacavir/lamivudine (\(n=4\)), zidovudine/lamivudine (\(n=8\)), tenofovir (\(n=3\)), tenofovir/abacavir (\(n=1\)) and lamivudine (\(n=1\)).

**Results**

**Patients**

The characteristics of the 80 included patients are summarized in Table 1. Most patients were male (94%) and in a good immunological and virological state. None of the included patients had a viral load \(>50\) copies/mL. The average CD4 cell count was 621 cells/mm\(^3\) (range 116–1560 cells/mm\(^3\)). For efavirenz, all patients were on the approved 600 mg once-daily dose regimen. For nevirapine, most patients (\(n=28\)) were on a once-daily 400 mg dose, 11 patients were on the approved 200 mg twice-daily dose and 1 patient was on a once-daily 500 mg dose. The most commonly used nucleoside analogue backbone was tenofovir/emtricitabine (\(n=58\)). Other nucleoside backbones were lamivudine/tenofovir (\(n=5\)), abacavir/lamivudine (\(n=4\)), zidovudine/lamivudine (\(n=8\)), tenofovir (\(n=3\)), tenofovir/abacavir (\(n=1\)) and lamivudine (\(n=1\)).
The relationships between nevirapine and efavirenz concentrations in DBS and plasma are plotted in Figure 1 and showed good correlations ($r^2 = 0.867$ and $0.972$ for nevirapine and efavirenz, respectively). The DBS concentrations of efavirenz were, however, 39.8% (SD 7.1%) lower than the corresponding plasma concentrations. For nevirapine, the DBS and corresponding plasma concentrations were almost identical. The linear relationship between theoretical nevirapine and efavirenz plasma concentrations (using the formula $[\text{DBS}_{\text{analyte}}](1 - \text{haematocrit}) \times f_{\text{bpp}} = \text{plasma}_{\text{analyte}}$ with patient-specific haematocrit) and measured plasma concentrations are plotted in Figure 2(a) and 2(b), respectively. The relationship almost equalled the line of true identity. When using mean haematocrit for males to calculate the theoretical plasma concentration, similar plots were seen ($r^2 = 0.867$ and $0.972$ for nevirapine and efavirenz, respectively). Figure 3 shows the Bland–Altman plots of both compounds using patient-specific and mean haematocrit for males. The mean difference in nevirapine and efavirenz concentrations between the theoretical plasma concentration using patient-specific haematocrit and measured plasma concentration was 0.29 mg/L (SD 1.35 mg/L) and 0.08 mg/L (SD 0.31 mg/L), respectively, whereas this difference was 0.43 mg/L (SD 0.31 mg/L) and 0.17 mg/L (SD 0.29 mg/L) when using the mean haematocrit for males. The mean differences between these two methods were significantly different for both nevirapine and efavirenz ($P = 0.007$ and $0.009$, respectively). Table 2 shows the discrepancies in categorization for efavirenz. In 10% ($n = 4$) of the cases, theoretical plasma concentrations were categorized differently from corresponding plasma concentrations.

### Discussion

In this study we demonstrated that DBS concentrations of nevirapine and efavirenz showed good agreement with plasma concentrations after correction for haematocrit and compound-specific plasma protein binding.

Previously, four studies evaluated the correlation between antiretroviral drug concentrations in DBS and plasma. Van Schooneveld et al. demonstrated in 48 individual patient samples that atazanavir DBS concentrations correlated well with plasma concentrations ($r^2 = 0.988$), although DBS concentrations were slightly lower (−10.8%) than plasma concentrations. Koal et al. evaluated 70 patient samples (lopinavir, atazanavir, ritonavir, saquinavir and efavirenz) for DBS and plasma concentrations. A summary correlation curve showed a good correlation ($r^2 = 0.9772$) for all compounds; however, DBS concentrations again were slightly lower (−15%) than the corresponding plasma concentrations. Meesters et al. showed the correlation between lopinavir and ritonavir in 19 DBS and plasma samples from children ($r^2 = 0.8487$ and $0.7679$, respectively). These DBS samples were, however, obtained from whole blood samples (not from a finger prick) and no bias estimation...
was performed. Our group previously showed that DBS concentrations of etravirine, darunavir, raltegravir and ritonavir were proportional to plasma concentrations obtained from cell preparation tubes.\(^9\) Thus, all previous authors reported that DBS and plasma concentrations correlated well, although the concentrations were not identical. This study showed similar results; nevirapine and efavirenz DBS concentrations correlated well with plasma concentrations, although DBS concentrations of efavirenz were 39.8% (SD 7.1%) lower than plasma concentrations. None of the previous authors investigated the mechanism behind the non-similarity between DBS and plasma concentrations. We, however, now show that the difference in DBS and plasma concentrations could very well be explained by compound-specific plasma protein binding and haematocrit using an adapted version of a formula previously proposed by Li and Tse.\(^9\)

The mean difference between measured and theoretical plasma concentrations using the measured haematocrit for nevirapine and efavirenz was 0.29 and 0.08 mg/L, respectively. This difference may have resulted in different clinical decisions in 10% of the efavirenz cases (Table 2). However, in clinical practice this difference may be less pronounced since the discrepancies between differently categorized concentrations were small. For example, in two of the four cases all efavirenz concentrations were around 1.0 mg/L (1.01, 0.68 and 0.89, 1.00 mg/L for the measured plasma concentrations and theoretical plasma concentration using patient-specific haematocrit, respectively). No dose adjustment is recommended with a measured plasma concentration of 1.01 mg/L according to categorization. However, since this concentration is at the boundary of acceptability, in clinical practice a dose increase may be advised if a specific patient is failing virologically despite adherence. Thus, despite different categorization of the measured and theoretical plasma concentrations, clinical practice in these patients may be similar.

The mean difference between measured plasma concentrations and theoretical plasma concentrations using mean haematocrit for males (0.43 and 0.17 mg/L for nevirapine and efavirenz, respectively) was significantly higher than the mean difference between measured plasma concentrations and theoretical plasma concentrations using measured haematocrit (P=0.007 and 0.009 for nevirapine and efavirenz, respectively). However, no difference in efavirenz categorization was found, showing the two methods performed similarly when used for TDM purposes.

A limitation of this study is that we did not investigate patient-specific plasma protein binding and binding to red blood cells. On average, efavirenz is highly bound (99.5%) to plasma proteins, mainly albumin, whereas binding of nevirapine to plasma proteins is ~60%.\(^8\) Inter-patient variability in albumin levels might result in inter-patient variability in plasma protein binding, thereby causing some bias. However, we investigated albumin levels in all patients and found albumin levels within the normal range (35–50 g/L) except for one patient (33 g/L). Thus, the variability in plasma protein binding of efavirenz and nevirapine in this population is thought to be minimal, thereby limiting potential bias. Additionally, the binding of nevirapine and efavirenz to red blood cells is thought to be limited. Binding of nevirapine and efavirenz to red blood cells may cause bias in the calculation of plasma concentrations from DBS concentrations. However, we showed that plasma concentrations could be adequately predicted by plasma protein binding and haematocrit. This indicates that preferential uptake in red blood cells was minimal, which can be expected from the relatively high plasma protein binding of both drugs. Another limitation is that our study population mainly included male patients. For calculation of the plasma concentrations we therefore used the mean haematocrit of males. Since the mean haematocrit of women and children is different, confirmation of our results in these populations is needed.

To conclude, the results of this study enable DBS sampling of nevirapine and efavirenz for TDM purposes. The theoretical plasma concentration can be calculated from DBS concentrations with the formula: \[ \text{DBS concentration} = \frac{\text{BDs}_{\text{analyte}}}{(1 - \text{haematocrit})} \times \text{f}_{\text{app}}. \]

Thus, for instance, an efavirenz (\(f_{\text{app}}=0.995\)) DBS concentration of 1.2 mg/L corresponds to a theoretical plasma concentration of 2.2 mg/L \((1.2/(1-0.45))\times0.995\). This opens up possibilities for TDM in special populations, such as children and pregnant women, where previously only limited sampling was allowed.

---

**Figure 2.** Theoretical plasma concentrations plotted against measured plasma concentrations for (a) nevirapine and (b) efavirenz. The broken line is the line of true identity.
Figure 3. Bland–Altman plots for (a) nevirapine and (b) efavirenz concentrations using measured haematocrit, and (c) nevirapine and (d) efavirenz concentrations using mean haematocrit of males. The continuous line is the mean and the broken lines represent the 95% CI (±2 SD).

Table 2. Comparison between the 40 samples categorized using theoretical efavirenz plasma concentrations and measured plasma concentrations

<table>
<thead>
<tr>
<th>Theoretical plasma levels using patient-specific haematocrit (mean male haematocrit)</th>
<th>&lt;1.0 (mg/L)</th>
<th>&gt;1.0 and &lt;4.0 (mg/L)</th>
<th>&gt;4.0 (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma levels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1.0 (mg/L)</td>
<td>2 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1.0 and &lt;4.0 (mg/L)</td>
<td>1 (1)</td>
<td>36 (36)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>&gt;4.0 (mg/L)</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>

Discrepancies in categorization are shown in bold.
due to ethical considerations, and in resource-limited settings, where equipment for plasma sampling is often not available. Evaluation of other antiretroviral drugs is currently ongoing.

Acknowledgements
We would like to acknowledge the assistance of the Department of Clinical Chemistry in patient recruitment.

Funding
This work was supported by internal funding.

Transparency declarations
None to declare.

References